

Gliotoxin Production in *Aspergillus fumigatus* Contributes to Host-Specific Differences in Virulence

Sara Spikes,¹ Ran Xu,¹ C. Kim Nguyen,¹ Georgios Chamilos,¹ Dimitrios P. Kontoyiannis,¹ Raymond H. Jacobson,² Daniele E. Ejzykiewicz,³ Lisa Y. Chiang,³ Scott G. Filler,^{3,4} and Gregory S. May¹

¹Division of Pathology and Laboratory Medicine and ²Department of Molecular Biology and Biochemistry, University of Texas, M. D. Anderson Cancer Center, Houston, Texas; ³Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, and ⁴David Geffen School of Medicine at UCLA, Los Angeles, California

Background. Gliotoxin is a epipolythiodioxopiperazine toxin that is made by the filamentous fungus *Aspergillus fumigatus*. Gliotoxin has a wide range of effects on metazoan cells in culture, including induction of apoptosis through inhibition of NF- κ B, and inhibition of superoxide production by phagocytes. These activities have led to the proposal that gliotoxin contributes to pathogenesis during invasive aspergillosis. We tested this hypothesis by creating isogenic strains of gliotoxin-producing and nonproducing strains.

Methods. We deleted *gliP*, the gene that encodes the nonribosomal peptide synthetase GliP. GliP catalyzes the first biosynthetic step in the synthesis of gliotoxin. We then tested for gliotoxin production and virulence in different animal models.

Results. Deletion of *gliP* resulted in strains that were wild type for growth, but they did not synthesize gliotoxin. Transformation of *gliP* deletion mutants with a full copy of *gliP* restored gliotoxin production. The *gliP* deletion strain had attenuated virulence in nonneutropenic mice immunosuppressed with corticosteroids, but had normal virulence in neutropenic mice. It also had reduced virulence in a *Drosophila melanogaster* model.

Conclusions. Gliotoxin only contributes to the virulence of *A. fumigatus* in nonneutropenic mice and in fruit flies with functional phagocytes. These results suggest that the principal targets of gliotoxin are neutrophils or other phagocytes.

Filamentous fungi produce an array of chemicals called secondary metabolites, which are not part of primary metabolism. These secondary metabolites include the following: antibiotics (e.g., penicillin), HMG-CoA reductase inhibitors (e.g., statins), and toxins (e.g., aflatoxin, fumonisin, and gliotoxin). Fungi that produce toxic secondary metabolites, also called mycotoxins, are referred to as toxigenic fungi or molds. Because of the vast array of chemicals produced by filamentous fungi, the biosynthesis of only a few, such as aflatoxin, and

penicillin, has been extensively studied and characterized. Gliotoxin is a member of the epipolythiodioxopiperazines, which are characterized by an internal disulfide bridge, a diketopiperazine ring, and an aromatic amino acid (phenylalanine) [1]. Gliotoxin synthesis is initiated by the condensation of serine and phenylalanine, a reaction that is catalyzed by a nonribosomal peptide synthetase (NRPS) [1]. Work on the synthesis of a related toxin, sirodesmin in *Leptosphaeria maculans*, led to the identification of a gene cluster in *Aspergillus fumigatus* that was thought likely to be responsible for gliotoxin synthesis because of gene similarities with the sirodesmin biosynthetic gene cluster [1–3]. This led to the identification of the *gliP* gene, the NRPS responsible for the first step in gliotoxin biosynthesis. Although the biological activities of gliotoxin are still under investigation, it is known that this molecule induces host-cell apoptosis [1, 3–5]. Additional activities of gliotoxin include inhibition of macrophage and polymorphonuclear cell function [1]; inhibition of NF- κ B, a transcriptional regulator of the host proinflammatory response; and inactivation of enzymes such as alcohol dehydroge-

Received 21 December 2006; accepted 2 October 2007; electronically published 16 January 2008.

Potential conflicts of interest: none reported.

Financial support: National Institutes of Health (grants AI051144 and AI052236 to G.S.M. and AI073829 to S.G.F.); National Institute of Allergy and Infectious Diseases (contract N01-AI-30041 to G.S.M. and S.G.F.). The Cancer Center Support Grant CA16672 is acknowledged for support of the DNA sequencing core facility used in these investigations.

Reprints or correspondence: Gregory S. May, Div. of Pathology and Laboratory Medicine, Unit 54, University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030 (gsmay@mdanderson.org).

The Journal of Infectious Diseases 2008; 197:479–86

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0022-1899/2008/19703-0022\$15.00

DOI: 10.1093/infdis/jin044

Table 1. Name and sequence of oligonucleotides used in these studies.

Primer Name	Sequence (5'→3')
attB1-An actin5' F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTTGGTCGTCGGTCTCTGCC
An actin5'-Hph R	GACAGACGTCGCGGTGAGTTCAGGCTTTTTCATGGTGTTTAGGGGTGGATTAGAATC
Hph-An actin3' F	CCCCAGCACTCGTCCGAGGGCAAAGGAATAGCTCTTGCGTTGTATATGGTCGTTG
attB2-An actin3' R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTACAAAATTTATCTTGATACTAGC
AfNRPS1del15'F	CTAGAACTGCAGACGGCGACC
AcHph3'F-NRPS15'R	TTGCTAGTTATACAAGATAAATTTGTACAGATGCAGAGCGTAGGGTTGAGC
AcHph5'R-NRPS13'F	ATCCTCGTGGGGCAGAGACCGACCAAGACCAGCTGCGGCAATCGTATCC
AfNRPS1del13'R	GTCCGACACGGGCTGGAGATC

nase, creatine kinase, and farnesyltransferase [1, 6]. Considering the prevalence of gliotoxin-producing fungi and the activities of the toxin, gliotoxin is an excellent candidate for a virulence factor [1, 7].

In this study, we analyzed the contribution that gliotoxin makes to the virulence of *A. fumigatus*. We generated isogenic *A. fumigatus* strains that were wild type and strains from which *gliP* had been deleted. We demonstrated that *gliP* deletion mutants fail to produce gliotoxin, whereas the wild-type and a *gliP*-complemented deletion mutant produced gliotoxin. We also determined that gliotoxin production correlates positively with virulence in a nonneutropenic mouse model of invasive pulmonary aspergillosis and a *Drosophila melanogaster* model of aspergillosis, but not in a neutropenic mouse model of pulmonary aspergillosis.

METHODS

A. fumigatus strains and growth media. *A. fumigatus* (Af293) was grown on minimal medium, complete medium, or yeast extract agar glucose (YAG) medium as described elsewhere [8, 9].

Construction of *gliP* deletion mutant. We modified a conventional hygromycin resistant marker (*hph*) for *A. fumigatus* transformation [10]. The promoter and terminator regions of the *A. nidulans* actin gene were polymerase chain reaction (PCR)–amplified from genomic DNA using attB1-An-actin5'F and Ac-actin5'-HphR; Hph-An-actin3'F, and attB2-An-actin3'R. The sequence of oligonucleotides used in these studies is provided in table 1. The primers were designed so that the 3'-end of the promoter and the 5'-end of the terminator contained 30 bp that overlap with the 5' and 3'-regions of *hph* open reading frame, respectively. The *hph* gene was released from an existing plasmid pCB1003 [10] by *HpaI* digestion and gel purified. The 3 DNA fragments were then annealed in a nonprimer PCR by use of YieldAce DNA polymerase (Stratagene) using the following thermal cycle program: 92°C for 2 min; 20 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 2 min; then 72°C for 7 min. Two μ L of this reaction product was used as a template in a PCR that used primers attB-An-actin5'F and attB-An-actin3'R to amplify the annealed whole construct with the YieldAce

(Stratagene). The final PCR product was purified with the Qia-gen Gel Extraction Kit (Qiagen) and cloned into vector pDONR 221 via BP recombination reaction (Invitrogen) to generate pDONR221-[AcHph].

To increase the efficiency of the desired homologous recombination, we incorporated a herpes simplex virus thymidine kinase (tk) gene as a negative selection marker. We obtained a plasmid, pPZtk [11], containing bidirectional tk genes flanked by the *A. nidulans* TrpC promoter and terminator (TrpC-tk). We released the TrpC-tk fragment from pPZtk by *EcoRI* digestion and cloned it into pCR vector, which was derived from pCR2.1 T-vector (Invitrogen), producing the plasmid pCR[TrpC-tk].

We replaced the first 2 kb of *gliP* with AcHph. Primers AfNRPS1del15'F and AcHph3'F-NRPS15'R were used to amplify a 2 kb region upstream of the start codon of the *gliP* gene from *A. fumigatus* genomic DNA. The 3' end of this amplicon contains the last 30 bp of the 3' end sequence of AcHph. Similarly, primers AcHph5'R-NRPS13'F and AfNRPS1del13'R were used to amplify the 2–4 kb section of the *gliP* coding region. The 5' end of the PCR fragment was attached with a 30 bp region overlapping with the AcHph 5' end sequence. The AcHph marker was the purified PCR product obtained during the construction of the plasmid pDONR221-[AcHph]. The 3 DNA fragments described above were annealed in a nonprimer PCR using ExTaq polymerase (Chemicon International) as described above. The 6 kb product was gel purified and used as template in a PCR with the AfNRPS1del15'F and AfNRPS1del13'R to amplify the full length product, as described above. This PCR product was cloned into pCR2.1 T-vector (Invitrogen) to obtain pCR2.1rc[AfNRPS1d3-AcHph]. The insert was released by *XbaI* digestion and cloned into the pCR[TrpC-tk] plasmid at *XbaI* site. This plasmid, pCR[TrpC-tk_ AfNRPS1d3-AcHph], was linearized with *BamHI* and transformed into Af293 [9, 12]. Transformants were selected on complete media with 200 μ g/mL hygromycin and 5 μ M fluorodeoxyuridine (FdU) (Sigma-Aldrich). Deletion mutants were identified by Southern blot analysis (figure 1) [13]. From this analysis, a number of independent *gliP* deletion mutants were identified and initially characterized. The Δ gliP3 strain was chosen for further characterization.

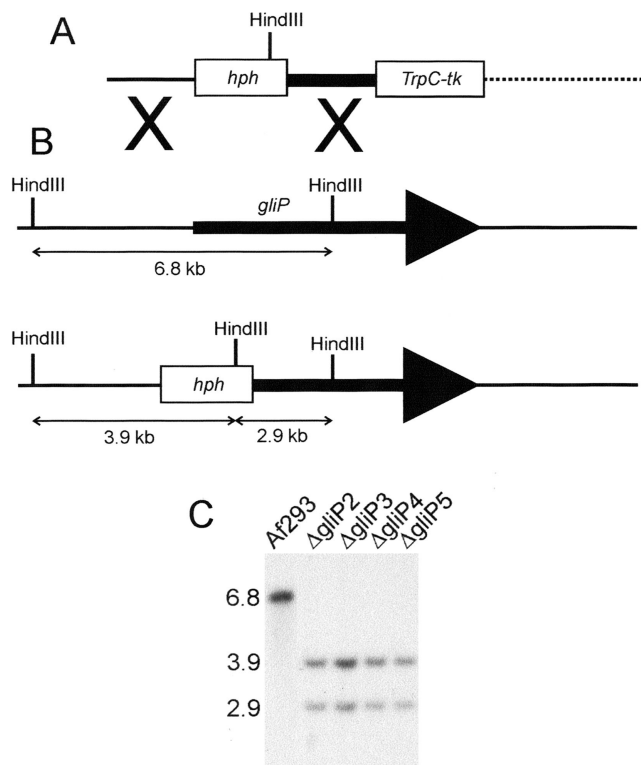


Figure 1. Deletion of *Aspergillus fumigatus* *gliP*. **A**, Structure of the linearized plasmid used for transformation of Af293 to obtain *gliP* deletion mutants. The linear DNA has the positive selective *hph* gene and the negative selective *TrpC-tk*. Correct integration of the linear DNA results in replacement of the first 2 kb of the *gliP* gene with *hph* and loss of the *TrpC-tk*. **B**, Graphical representation of the *gliP* genomic locus from the wild-type and the deletion mutant are shown along with the expected size of bands for digestion with *HindIII*. **C**, Autoradiograph of genomic Southern blot analysis of wild-type and *gliP* deletion mutants probed with the plasmid used to create the deletion mutant. The 6.8 kb predicted *HindIII* band of hybridization for wild-type and the 2.9 and 3.9 kb bands predicted for the deletion mutants are clearly evident.

Cloning of *gliP* and complementation of the deletion strains. To restore *gliP* function to the deletion mutant, we amplified *gliP* from wild-type genomic DNA by use of the oligonucleotides XbaI_{gliP}F and XbaI_{gliP}R, which introduced *Xba*I sites flanking the amplified DNA. The amplified DNA was digested with *Xba*I and cloned into the pBC-phleo [14] digested with *Xba*I and dephosphorylated with calf intestinal alkaline phosphatase to produce pBC-phleo-*gliP*. This plasmid was then used to transform the *gliP* deletion strain Δ *gliP*3 to phleomycin resistance by selection on complete media plus 50 μ g/mL phleomycin. Phleomycin resistant colonies were then tested for production of gliotoxin to identify complemented strains. A Δ *gliP*3 + *gliP* strain was identified and used for further study.

Extraction and detection by liquid chromatography–mass spectrometry of gliotoxin. Gliotoxin was isolated from culture medium after 72 h growth at 37°C by extracting an equal volume (1–20 mL) of spent medium with chloroform for 30 min at room

temperature. The chloroform was evaporated to dryness to concentrate the metabolites that were subsequently dissolved in 10–100 μ L of methanol. The presence or absence of gliotoxin was then determined using high-performance liquid chromatography–mass spectrometry. A gliotoxin standard was run to ensure identity of the product and to provide an estimate of the amount of gliotoxin in the culture medium.

One μ L of extract was loaded onto a 1 \times 150 mm analytical reversed phase column (Higgins Cliepeous C18) equilibrated with 0.02% trifluoroacetic acid in 28% acetonitrile/H₂O. The samples were eluted isocratically while following the UV absorbance at 254 nm and the signal from an Agilent LC-MSD Trap SL mass spectrometer. Mass spectra were collected in positive ion mode, scanning between 100 and 500 m/z. To assay for the presence of gliotoxin in the samples, an extracted ion current was calculated that consisted of 3 characteristic positive ions that resulted from partial fragmentation or degradation of the gliotoxin molecule (parent ion [327 amu], dethiogliotoxin [263 amu], and a further neutral loss of H₂O from dethiogliotoxin [245 amu]). Injection of 1 μ L of a gliotoxin standard (Sigma-Aldrich) was found to elute between 7–8 min after injection, as indicated by readily apparent peaks in both the UV and extracted ion current chromatograms and characteristic gliotoxin ions (figure 2). Unknowns corresponding to the strains Af293, Δ *gliP*, and Δ *gliP* complemented (Δ *gliP* + *gliP*) strain were injected and assayed as above. The extracts from Af293 and Δ *gliP* + *gliP* strain were both found to contain gliotoxin as evident by peaks in the UV and extracted ion current chromatograms, while the Δ *gliP* extract contained no detectable gliotoxin.

Assessment of virulence in mouse models of invasive aspergillosis. Three different mouse models of invasive pulmonary aspergillosis were used to assess the virulence of the various strains. The first model was an inhalation model that used a neutropenic host, as described elsewhere [15, 16]. Eighteen mice were infected for 1 h in an inhalation chamber system [15–17] by aerosolizing 12 mL of a suspension of the Af293 conidia, Δ *gliP*3, or Δ *gliP*3 + *gliP* strains containing 10⁹ conidia/mL [15]. From each group, 3 mice were sacrificed immediately after removal from the chamber to confirm delivery of conidia to the lungs. Mice were monitored daily for 14 days. Mice that appeared moribund were euthanized. The second model, which involved immunosuppression with steroids, used a modification of the protocol described by Pardo et al. [5]. Mice were treated with cortisone acetate (5 mg per mouse, administered subcutaneously) every other day, beginning on day –4 relative to infection and ending on day +4, for a total of 5 doses. For inoculation, the mice were anesthetized with isoflurane, after which 5 \times 10⁶ conidia in 25 μ L of PBS containing 0.1% Tween was instilled intranasally. Ten mice were infected with each strain of *A. fumigatus*. The third model used the same steroid immunosuppression regimen as in the intranasal model, but it used 10 mg cortisone acetate followed by

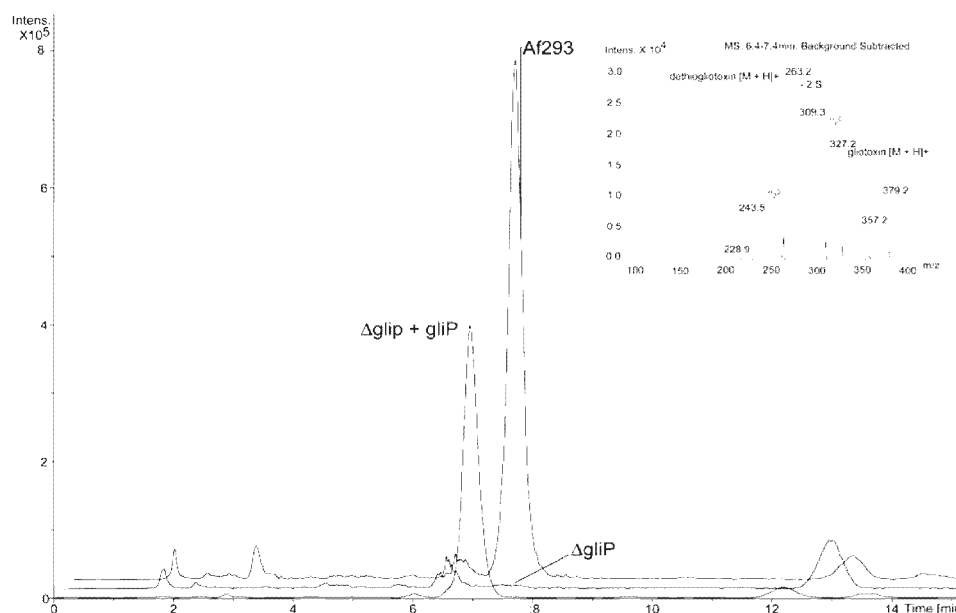


Figure 2. In vitro gliotoxin production by the various strains. Overlaid extracted ion chromatographs (extracted ion currents 327.2, 263.2, and 245.2) between 0 and 14 min after injection for strains Af293, Δ gliP, and Δ gliP+gliP strains. Separation conditions used were isocratic with 28% acetonitrile/water + 0.02% trifluoroacetic acid, 50 μ L/min on a 1 \times 150 mm reversed phase column (Higgins Cliepeous C18). No peak for gliotoxin is observable in the Δ gliP chromatogram, nor are gliotoxin ions present in the corresponding portion of the MS spectra. Both Af293 and Δ gliP+gliP strains show a peak between 6.5–8.0 min that corresponds to gliotoxin. The inset at the upper right shows an averaged mass spectrum (MS) that includes scans between 6.4–7.4 min from the Δ gliP+gliP strain extract that correspond to the gliotoxin peak and reveals characteristic ions for the [M+H]⁺ ions of gliotoxin and dethiogliotoxin.

infection by use of the inhalation chamber method. Eight mice were infected with either the wild-type strain or the Δ gliP3 mutant. The survival rate experiments with the first 2 mouse models were repeated twice and the results of similar experiments were combined.

Two separate methods were used to determine the pulmonary fungal burden of the mice infected with *A. fumigatus* strains Af293, Δ gliP3, or Δ gliP3 + gliP. The mice were immunosuppressed with cortisone acetate and infected intranasally with *A. fumigatus*, as described above. They were sacrificed on day +3, relative to infection. From 8 to 9 mice per strain, the lungs were harvested, weighed, homogenized, and quantitatively cultured, as described elsewhere [18]. Also, for each strain tested, the lungs from 2 mice were fixed in zinc-buffered formalin and embedded in paraffin. Thin sections were cut and stained with periodic acid–Schiff. Images were captured using a Leica DFC320 CCD camera on a Nikon Microphot-SA, microscope using a 10X objective lens. The area of the sites that contained fungal hyphae with neutrophil infiltrate was determined by use of Image-Pro Express (version 4.5, Media Cybernetics). Briefly, the software was used to outline lesions and the outlined area was determined for 7–10 sites of infection for each strain tested. In addition, we determined the number of lesions per lung for each pair of mice that had been infected with 1 of the 3 strains. All studies were approved by the institutional animal care and use committee, according to the National Institutes of Health guidelines for animal housing and care.

Injection assay with Toll-deficient (TL) *D. melanogaster*.

The dorsal side of the thorax of 25–30 CO₂-anesthetized flies was punctured with a thin (0.25 mm), sterile needle that had been dipped in a concentrated solution of *A. fumigatus* conidia (10⁸ per mL) [19, 20]. Flies that died within 3 h of the injection (<5%) were considered to have died as a result of the procedure and were not included in the survival rate analysis. Adult flies 2–4 days old were housed at 29°C, at which *Tl* susceptibility to microbial challenge is maximal [21]. The flies were transferred every 2 days to new medium, and survival rate was assessed daily for 8 days. The experiments were performed 3 times. The conidial inoculates introduced into flies were quantified by transferring conidia from the tip of a needle previously dipped in 10⁸ conidia/mL solution to 1 mL of sterile saline. Serial dilutions of the solution were plated (100 μ L) in triplicate on yeast extract glucose plates at 37°C, and the colony-forming units (cfu) were counted after 48 h.

Statistics. The survival rate of mice or flies infected with the various strains of *A. fumigatus* was analyzed using the log-rank test. Differences in pulmonary fungal burden among the groups of mice were analyzed by the Wilcoxon rank sum test.

RESULTS

Isolation and characterization of gliP deletion mutants. *gliP* deletion mutants were isolated following transformation with a positive (hygromycin) and negative (TK/FdU) linear construct

pCR-[TrpC-tk AfNRPS1d3-AcHph] (figure 1A). Deletion mutants were identified by Southern blot analysis of genomic DNA probed with the transforming vector (figure 1). The growth and conidiation of all *gliP* deletion mutants was indistinguishable from wild type at 25°C, 30°C, 37°C, and 42°C on a variety of growth media (data not shown). The Δ gliP3 strain was transformed with pBC-phleo-gliP to complement the *gliP* deletion mutation. We then characterized gliotoxin production in the wild-type parental strain, Δ gliP3, and Δ gliP3+gliP strains by high-performance liquid chromatography–mass spectrometry analysis (figure 2). Gliotoxin was readily detected in the wild-type parental strain and the Δ gliP3+gliP strain, and it was absent in the Δ gliP3 strain, results that were consistent with *gliP* encoding the enzyme for the first step in the biosynthesis of gliotoxin (figure 2).

Gliotoxin contributes to the virulence of *A. fumigatus* in nonneutropenic animal hosts. The contribution of gliotoxin to the virulence of *A. fumigatus* was tested in 2 different mouse models of invasive aspergillosis. Conidia from the Af293, Δ gliP3, and Δ gliP3+gliP strains were used to infect mice that were neutropenic and had been treated with corticosteroids [16]. In this model, the survival rate of mice infected with the Δ gliP3 deletion mutant was similar to that of mice infected with Af293 or the Δ gliP3+gliP strain ($P = .54$) (figure 3A). Therefore, gliotoxin production did not significantly contribute to the virulence of *A. fumigatus* in this model.

In the neutropenic mice, very few leukocytes were present at foci of *A. fumigatus* infection during the first 6 days of infection [16]. We reasoned that if gliotoxin's main effect was on neutrophils, this effect might not be apparent in neutropenic mice. Therefore, we also assessed the virulence of the different strains in nonneutropenic mice that had been immunosuppressed with corticosteroids. Because these mice were less immunosuppressed, a higher inoculum was required to induce lethal invasive pulmonary aspergillosis. We found that mice infected with the Δ gliP3 deletion mutant survived longer than did mice infected with Af293 or the Δ gliP3+gliP strain (figure 3B).

Microscopic examination of the lungs of the corticosteroid-treated mice after 3 days of infection revealed large areas of bronchopneumonia that consisted of hyphae surrounded by an extensive neutrophilic inflammatory infiltrate. The number and size of these areas of bronchopneumonia were similar in mice infected with all 3 strains (figure 4A–4C; table 2). Interestingly, the neutrophils in the lesions of the mice infected with either Af293 or the Δ gliP3+gliP strain had extensive nuclear fragmentation, suggesting that they had undergone apoptosis and/or necrosis (figure 4D and 4F). In contrast, the neutrophils present in the pulmonary lesions of mice infected with the Δ gliP3 deletion mutant appeared to be largely intact (figure 4E). Collectively, these results indicate that gliotoxin has toxic effects on neutrophils, and this toxicity contributes to *A. fumigatus* virulence in nonneutropenic mice.

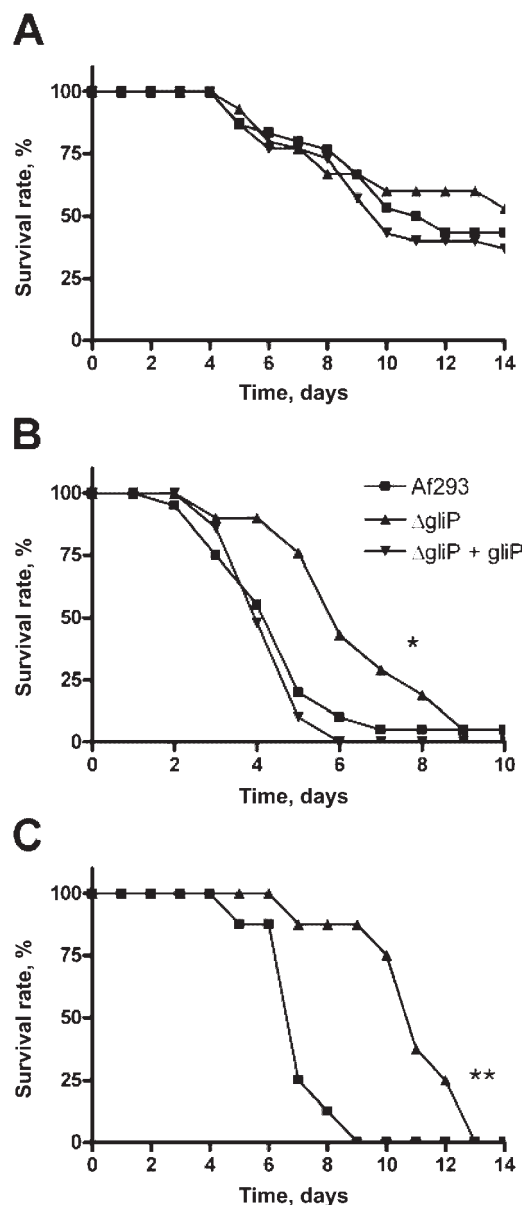


Figure 3. Comparison of virulence for strains in 2 different mouse models of invasive pulmonary aspergillosis. *A*, Survival rate of mice that were immunosuppressed with cortisone acetate and cyclophosphamide, and then inoculated by placing them in a chamber containing an aerosol of *Aspergillus fumigatus* conidia. Data are the combined results of 2 independent experiments (30 mice per strain). *B*, Survival rate of mice that were immunosuppressed with cortisone acetate alone and then inoculated intranasally with *A. fumigatus* conidia. Data are the combined results from 2 independent experiments (20 mice per strain). * $P \leq .004$, compared with mice infected with either the wild-type strain or the Δ gliP+gliP complemented strain. *C*, Survival rate of mice that were immunosuppressed with cortisone acetate alone and then inoculated by placing them in a chamber containing an aerosol of *A. fumigatus* conidia (8 mice per strain). ** $P \leq .002$ for Δ gliP, compared with wild-type. The Δ gliP+gliP complemented strain was not tested in this third experiment because it gave effectively wild-type results in all prior tests.

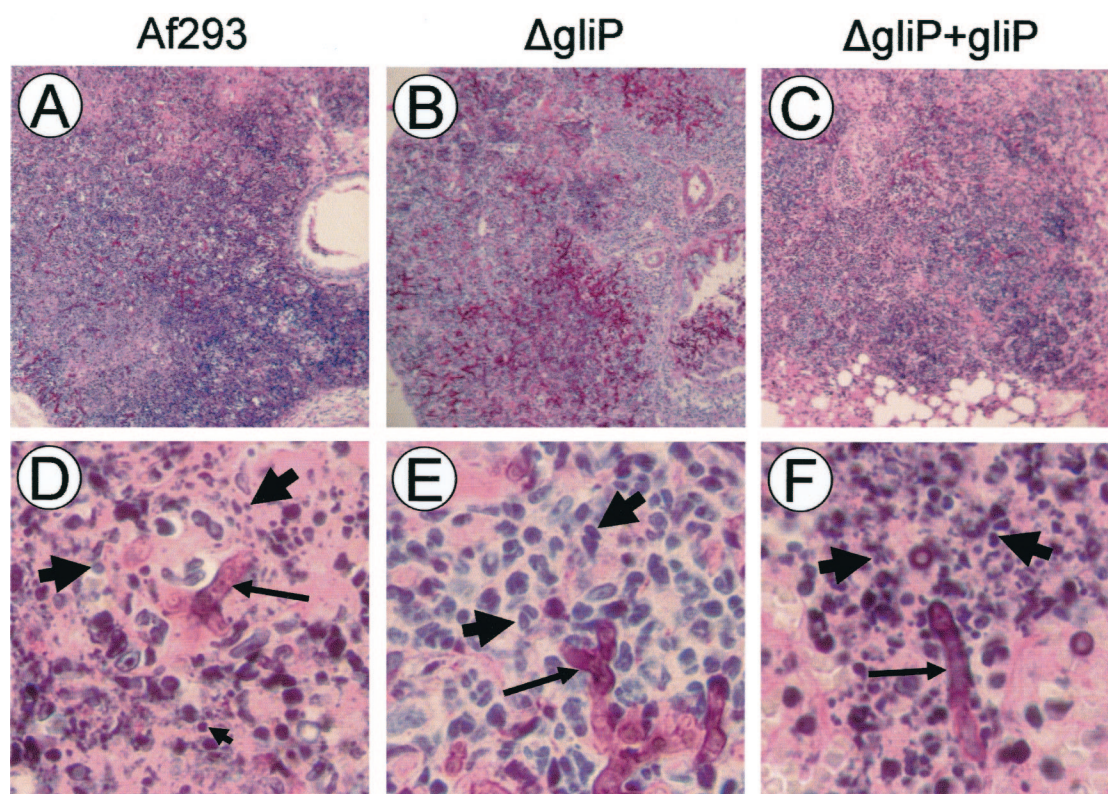


Figure 4. Size of fungal lesions and extent of neutrophil destruction in the lungs of mice infected with the various strains of *Aspergillus fumigatus*. Mice were immunosuppressed with cortisone acetate and then infected intranasally with the strains indicated. The images are of representative sections of the lungs stained with periodic acid-Schiff stain after 3 days of infection. A–C, 100× images of the lungs showing similar-sized areas of bronchopneumonia. D–F, 1000× images of fungal lesions showing the neutrophilic infiltrate around the *A. fumigatus* hyphae. Note that the nuclei of neutrophils adjacent to hyphae of the Δ gliP strain are largely intact, whereas the nuclei of neutrophils adjacent to hyphae of the wild-type and Δ gliP+gliP strains are degenerating. Thick arrows indicate the neutrophils; thin arrows indicate the hyphae.

The pulmonary fungal burden of these mice was determined by quantitative culture. After 3 days of infection, the lungs of mice infected with Af293, Δ gliP3, and Δ gliP3+gliP contained a median of 5.8 log cfu/g of tissue (interquartile range, 0.2–0.1), 6.1 log cfu/g of tissue (interquartile range, 0–0.1), and 5.9 log cfu/g of tissue (interquartile range, 0–0.1 log cfu/g of tissue), respectively. Although, the pulmonary burden of mice infected with the Δ gliP3 strain was significantly greater than that of mice infected with the other 2 strains ($P < .01$), this difference was

too small to be biologically significant. These data suggest that the improved survival rate of the mice infected with the Δ gliP3 strain may have been the result of reduced pulmonary damage (i.e., from degrading neutrophils), rather than a decrease in pulmonary fungal burden.

To verify that the reduction in virulence of the Δ gliP3 deletion mutant was the result of the presence of neutrophils rather than the result of the intranasal route of inoculation, we compared the virulence of this strain with that of the wild-type strain in mice that were immunosuppressed with high-dose cortisone acetate and inoculated in the aerosol chamber. Mice infected with the Δ gliP3 deletion mutant survived significantly longer than mice infected with the wild-type strain (figure 3C). Therefore, gliotoxin is necessary for the full virulence of *A. fumigatus* in non-neutropenic mice.

We further assessed the importance of gliotoxin to virulence in *A. fumigatus* using a *D. melanogaster* model of infection, as an alternative and complement to the mouse model [20]. We previously showed that *T1* mutant flies are susceptible to lethal infection with wild-type Af293, whereas wild-type flies are not [20]. Flies infected with the wild-type parental strain or the

Table 2. Size and number of fungal lesions, according to strain.

Strain	No. of lesions measured	Lesion size, μm^2	Lesions per lung, no. ^a
Af293	10	$1.1 \times 10^6 \pm 0.7 \times 10^6$	10.8 ± 1.9
Δ gliP3	9	$1.3 \times 10^6 \pm 0.9 \times 10^6$	15.25 ± 10.9
Δ gliP3 + gliP	7	$2.7 \times 10^6 \pm 3.8 \times 10^6$	11.5 ± 1.7

NOTE. These data are the averages for 2 sections each from the lungs of 2 different animals. Values are means \pm SD.

^a Four lungs were analyzed for each strain.

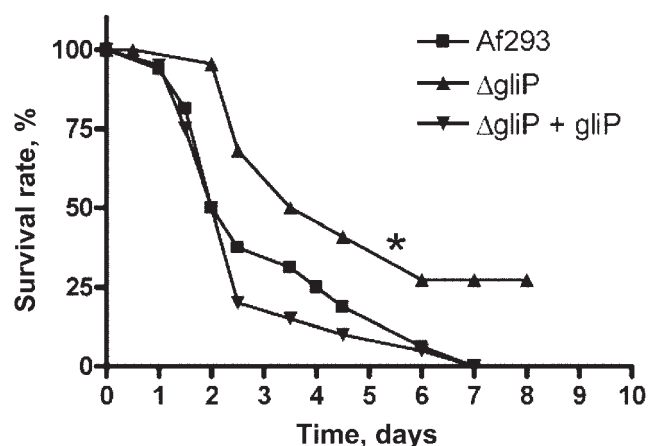


Figure 5. Comparison of virulence for the wild-type parental strain, Af293, Δ gliP, and Δ gliP+gliP strains in Toll-deficient *Drosophila melanogaster*. Groups of 25–30 flies were infected as described in Methods, and then the survival rate was followed over a period of 8 days. * $P \leq .005$, compared with mice infected with either the wild-type strain or the Δ gliP+gliP complemented strain.

Δ gliP+gliP strain began to succumb after 1 day and there was a rapid decrease in the survival rate over the ensuing 2 days. By day 7, all flies infected with the gliotoxin-producing strains had died. In contrast, the flies infected with the Δ gliP3 deletion mutant survived significantly longer than flies infected with the wild-type or Δ gliP3+gliP strains (figure 5). All flies infected with the Δ gliP strain survived for the first 2 days, and 30% of these flies survived until the end of the experiment. These results indicate that gliotoxin is required for maximal virulence in this model.

DISCUSSION

The condensation of serine and phenylalanine catalyzed by the NRPS GliP is the first step in gliotoxin biosynthesis. We constructed deletion strains of *gliP* that were phenotypically wild-type for growth in vitro, compared with the parental strain Af293, but the mutant did not to produce gliotoxin, which confirmed that GliP is required for gliotoxin biosynthesis. The *gliP* deletion mutant had significantly reduced virulence, compared with the wild-type parental strain and Δ gliP+gliP strain in 2 nonneutropenic mouse models of pulmonary aspergillosis. However, the *gliP* deletion mutant had virulence comparable to the isogenic wild-type strain in neutropenic mice. Other investigators have also found that *gliP* is dispensable for *A. fumigatus* virulence in neutropenic mice [22, 23]. Similarly, another group found that a mutant that lacked *gliZ*, which encodes a transcription factor important for gliotoxin production, had normal virulence in a neutropenic mouse model [4]. Collectively, these results indicate that gliotoxin only contributes to the virulence of *A. fumigatus* in nonneutropenic mice. Thus, the immunosuppressive effects of gliotoxin may not have been apparent in a neutropenic mouse model.

One explanation for the finding that gliotoxin is important for virulence only in nonneutropenic mice is that neutrophils are one of the principal host cell targets of gliotoxin, and thus need to be present for this toxin to have a significant effect on the host's defense against *A. fumigatus*. Consistent with this model, it was notable that mice infected with the *gliP* deletion mutant had reduced neutrophil fragmentation at foci of *A. fumigatus* infection, compared with mice infected with gliotoxin-producing strains. It is likely that the reduction in neutrophil death in the mice infected with the *gliP* deletion mutant was the result of decreased apoptosis. Bok et al. [4] found that culture supernatants of wild-type *A. fumigatus* induced apoptosis of neutrophils in vitro. However, culture supernatants of a *gliZ* deletion mutant did not contain gliotoxin and did not induce apoptosis in neutrophils.

It was notable that even though nonneutropenic mice infected with the Δ gliP strain lived significantly longer than mice infected with the wild-type strain, the pulmonary fungal burden of mice infected with the Δ gliP strain was at least as high as that of mice infected with the wild-type strain. These results suggest that even though there were more intact neutrophils in the pulmonary lesions of mice infected with the Δ gliP strain, these neutrophils were apparently unable to clear the organisms from the lungs. It has been suggested that mice that are immunosuppressed with corticosteroids alone die from invasive pulmonary aspergillosis because of an overexuberant inflammatory response [24]. Our results suggest that the effects of gliotoxin on neutrophils, and perhaps other leukocytes, may contribute to this lethal immune dysregulation.

We also found that the *gliP* deletion mutant displayed reduced virulence in flies when compared to wild-type or Δ gliP+gliP strains. The attenuated virulence of the *gliP* deletion mutant in *D. melanogaster* likely reflects increased susceptibility of the fly immune system to the immunosuppressive effects of gliotoxin or a difference in the degree of immunosuppression, compared with the neutropenic mouse model. Although the flies lack neutrophils, they do have functional circulating phagocytic cells [21]. Thus, we favor the idea that these phagocytic cells were one of the main targets of gliotoxin. However, it is also possible that the internal milieu of the fly stimulated greater gliotoxin production by the wild-type strain than did the environment of the mouse lungs.

In summary, we propose that gliotoxin production by *A. fumigatus* does not contribute significantly to the virulence of this fungus in neutropenic mice, but does contribute to virulence in an immunosuppressed host that retains phagocytic cell functions, such as in steroid-treated mice or insect models. Gliotoxin production in *A. fumigatus* may provide the fungus with a selective advantage in its normal ecological niche, in which it competes with an array of other microorganisms, but the role of gliotoxin in virulence in an animal

host is dependent on the degree to which the animal host is immunosuppressed.

Acknowledgments

We thank Angela Romans, Guiyu Jiang, Nathaniel D Alberts, and Norma Solis for their excellent technical support of this project.

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